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Salivary S100A8/A9 in Sjögren's syndrome accompanied by lymphoma

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Abstract

Background

Sjögren's syndrome (SS) is an autoimmune inflammatory disease that affects the exocrine glands. The absence of early diagnostic markers contributes to delays in its diagnosis. Identification of changes in the protein profile of saliva is considered one of the promising strategies for the discovery of new biomarkers for SS.

Objective

To identify salivary protein biomarkers with potential for use in discriminating between different lymphoma risk sub-groups of SS.

Method

Parotid and whole mouth saliva samples were collected from SS patients, including those in sub-groups at higher risk of developing or with confirmed lymphoma, non-SS sicca disease controls and healthy subjects. An initial proteomics analysis by mass spectrometry (LCMSMS) identified S100A8/A9 as a biomarker and was followed by validation with an enzyme linked immunosorbent assay (ELISA).

Results

Significant differences were found in levels of S100A8/A9 in parotid saliva but not whole mouth saliva between SS patients compared with healthy and disease control subjects ($p=0.001$ and 0.031respectively). Sub-groups of SS patients based on lymphoma risk showed significant differences in salivary levels of S100A8/A9.

Conclusion

The results suggest that salivary levels of S100A8/A9 can aid in differentiating between SS, disease control and healthy control subjects, especially the sub-groups of SS with lymphoma or at higher risk of lymphoma.

Introduction

Sjögren's syndrome (SS), also known as autoimmune exocrinopathy and autoimmune epitheliitis, is a systemic, chronic, inflammatory, autoimmune disease that affects the exocrine glands, particularly the salivary and lacrimal glands. The pathogenesis of the disease remains uncertain and to date no universally effective therapy is available. Clinically, dry mouth (stomatitis sicca or xerostomia), dry eyes (keratoconjunctivitis sicca (KCS) or xerophthalmia) and fatigue with /without inflammatory arthritis are among the most common manifestations[1].

The absence of early diagnostic markers contributes to delays in the diagnosis of SS and there is a well-recognized need for the identification and validation of biomarkers to be used in diagnosis, prognostic assessments, as a research tool and probably to identify novel targets for therapeutic intervention in SS. The evaluation of the composition of saliva, which can be non-invasively collected, could provide biomarkers for SS[2]. Changes in salivary proteins can reflect the pathogenesis of SS, since salivary glands are the major source of salivary proteins and a major site of autoimmune destruction; saliva is therefore an appropriate body fluid for biomarkers of SS[3]. There is a 1000-fold increased risk of lymphoma development in parotid glands from SS patients[4]. Parotid saliva is therefore a valuable substrate that might be used to differentiate between different sub-groups of SS patients; those at higher risk of developing mucosa associated lymphoid tissue lymphoma (MALT-L) and those who have developed MALT-L.

Since the first proteomic study on parotid saliva of SS patients in 1999 by Beeley and Khoo[5], salivary proteomics has remained one of the most promising approaches to human disease biomarker identification with the advantage of being non-invasively sampled. Several proteomic studies have shown differential protein expression in the saliva of SS patients and healthy control subjects[6,7] and some studies have validated candidate markers[8]. A few studies have performed proteomics analysis of saliva samples from lymphoma patients[9,10].

In the present study, it was hypothesized that SS related proteins exist in human saliva and could be used to discriminate SS patients from control subjects. Furthermore, identified salivary biomarkers could be useful in identifying a sub-group of SS who may be at higher risk of developing or who already have developed lymphoma. In order to

address the study aims a proteomic analysis of parotid saliva samples from healthy subjects, SS patients and SS patients at higher risk of developing lymphoma was undertaken and potential protein biomarkers identified. A candidate biomarker (S100A8/A9 heterodimer) was selected and validated by immunoassay of larger groups of samples including disease (dry mouth) control subjects and SS patients who have developed lymphoma. The marker was further tested on whole mouth saliva (WMS) and differences between both types of saliva were compared.

Materials and methods

Study group

The protocol for this study was reviewed and approved by the National Research Ethics Service (NRES) Committee (11/LO/1121). Patients with symptomatic dry mouth attending Guy's and St. Thomas's Hospital Oral Medicine department (GSTT NHS Foundation Trust) were included. Recruited patients were originally classified using the American-European Consensus Group (AECG) criteria[11]. Later patients were re-classified according to the ACR criteria[12] and some were included as SS patients in the present study (total n=51; mean age ±SEM, 52.3±1.6). Sjögren's syndrome patients were further sub-grouped into three groups:

a) SS-M patients who had been diagnosed with a MALT lymphoma (M), which had been confirmed by histopathological assessment of biopsies of parotid glands, minor salivary glands or submandibular gland, (n=14; mean age ±SEM, 53.7± 2.6).

b) SS at risk group: SS patients classified as high risk of developing MALT-L (SS-HR, n=18; mean age ±SEM, 51.9± 3), on the basis of three or more markers of severe SS (parotid enlargement, cryoglobulinaemic vasculitis, hypocomplementaemia, cryoglobulinaemia, high focus score, germinal centre in their biopsy and previous lymphoma), and SS-LR classified as low risk patients with less than three markers but could be associated with other generalised inflammatory factors -including raised β2 microglobulin levels, lymphopenia and hypergammaglobulinaemia[13]. SS-LR (n=19; mean age ±SEM, 51.5 ±2.7). c) SS sub-group (n=19; mean age ±SEM, 51.5 ±2.7) who were not considered at risk of developing MALT nor developed it.

Nineteen of the 51 SS patients were determined to have another autoimmune disease (e.g. rheumatoid arthritis, systemic lupus erythematosus and scleroderma, see supplementary material Table S1. Dry mouth patients complaining of xerostomia with hyposalivation, with a negative serology test for SS while having non- specific sialadenitis on their biopsy results and confirmed nodal osteoarthritis were diagnosed as SNOX [14] and were included as a disease control patient group (n=14; mean age ±SEM, 62.7±2.6). A further control group was formed from healthy subjects not taking medication, with no complaints of oral or ocular dryness and no oral mucosal diseases (n=18; mean age ±SEM, 50.7±2.6).

Collection of parotid and whole mouth salivas

Unstimulated whole mouth saliva (WMS) samples were collected over ten minutes followed by stimulated parotid saliva samples over 10 minutes. All saliva samples were immediately placed on ice and transferred to the laboratory where they were processed by centrifugation at 9500 g for 10 minutes (WMS) and 5 minutes (parotid saliva). Samples were split into a number of aliquots and stored at -80 °C until required. None of the aliquots were used more than once per assay. A minimum of 1hr fasting preceded all sample collections, which were undertaken between 09.00-12.00hr. Detailed protocols of saliva collection, flow rates and total protein concentrations (Bicinchoninic Acid assay; Thermo Scientific, Rockford, Illinois, USA) are described in supplementary material 2, 3 &4.

Proteomic analysis of parotid saliva

A pilot proteomics analysis was performed on parotid salivas from SS patients in order to identify potential biomarkers associated with parotid gland involvement and an increased risk of developing MALT-L. Parotid salivas from healthy subjects (n=2), SS patients (n=2) and SS patients at risk of developing MALT-L (n=2) were subjected to a proteomics analysis using LDS gel electrophoresis followed by trypsin digestion, peptide separation and mass spectrometry (LCMSMS) (see Supplementary material). The final selection of upregulated proteins was based on a ≥2 fold increase in both diseased samples (SS and SS at risk of MALT-L) when compared with healthy controls then in SS at risk of MALT-L when compared with SS (Supplementary material 5).

Enzyme linked immunosorbent assay (ELISA)

Commercially available pre-coated plates (Quantikine ELISA Kit, R&D Systems, Minneapolis, MN), (cat. no. DS8900) for S100A8/S100A9 heterodimer were used for both parotid saliva and WMS. The samples were thawed and diluted at an optimised dilution factor of 1/500 for WMS and 1/200 for parotid saliva. Standards and samples were loaded in duplicate. Incubations were done at room temperature on a horizontal orbital microplate shaker. All materials were supplied with the kit and the manufacturer's instructions were followed.

Data analysis

All results were exported to Windows® Excel 2007 spread sheets, statistical analyses for ELISA data were carried out using SPSS (IBM Corporation) and GraphPad Prism® 6 (GraphPad Software Inc., California, USA) was used for the graphical presentation. The Kruskal-Wallis test followed by Dunn's post-hoc test, was used to determine the differences between groups when comparing the mean amount of S100A8/S100A9 present in the salivary samples of the 5 different groups of participants. A study with 80% power and an effect size of 0.42 was determined to require a total sample of 75 (15 per group) for comparison of the protein levels at a 5% level of significance using a two-tailed test (G*power version 3.1.5 software). The median and quartiles were used as estimates of central tendency and dispersion. The significance level was set to $p<0.05$.

Results

Salivary flow rates and total protein concentrations

Parotid saliva samples were collected from 83 (100%) subjects while WMS was collected from 56/83 (68%). Insufficient samples were mostly from the patient groups; SS-LR 11/19 (57%), SS-HR 8/18 (44%), SS-M 7/14 (50%) and SNOX 12/14 (85%). WMS samples were collected from all 18 of the healthy control group. There were statistically significant reductions in the SS group compared to control group as well as for some of its sub-groups regarding flow rate and total protein concentration in WMS but parotid saliva only showed a difference in flow rate between the healthy control and SS (M) groups (Figure 1).

Proteomics analysis of parotid saliva

Four upregulated proteins; Actin cytoplasmic 2, Ig γ-1chain C region, S100-A8, and S100-A9 were finally selected as candidate biomarkers (Supplementary material Fig. S1). S100-A9 was not present in the healthy control subject sample in the first run while present in both disease samples. When comparing both disease samples of SS at MALT-L risk and SS only patients, both S100-A8 and S100-A9 were increased by two fold in both runs (Figure 2).

Salivary S100A8/A9 levels

Parotid saliva

The median concentration (Q1-Q3) of S100A8/A9 in parotid saliva from overall SS patients was 743.1 (91- 3526) ng/mL, which was significantly higher than concentrations in healthy (31.9; 0- 273.2 ng/mL) and disease control (208.9; 0- 265.3 ng/mL) subjects (Figure 3A). The median concentration of S100A8/A9 in the whole group of SS patients was 506 (84.6- 1031) ng/mL. The mean concentrations were statistically significantly higher in SS-HR patients (666.8; 131.2- 5642 ng/mL) and SS-M patients (937.4; 90- 4566 ng/mL) compared with healthy controls (Figure 3B). The values in the SS-LR group were not significantly raised.

Two patients in the SS-HR group presented with unilateral parotid swelling and the S100A8/A9 levels were compared between salivas from the right and left parotid

glands of the same patient (n=2). Higher levels were detected in salivas from the swollen side for both patients (Supplementary material, Table S2). The higher S100A8/A9 levels were confirmed when the samples were assayed a second time.

Whole mouth saliva

The median (Q1-Q3) S100A8/A9 concentration was twenty fold higher in WMS from the overall SS group (16628; 3165 - 20184 ng/mL) compared with parotid saliva. There was no statistically significant difference with concentrations of S100A8/A9 in WMS from the healthy (8457; 687.3- 17154 ng/mL) and disease control (3857; 2418-8697 ng/mL) groups (Figure 3C). However, SS sub-groups did show significantly higher levels, also the median concentration in SS-M patients (22545; 16032- 28096 ng/mL) was higher than the healthy control group (p=0.046), SNOX group (p=0.009) and the SS sub-group (5244; 1550- 17225 ng/mL) (p=0.049; Figure 3D).

In both parotid and WMS, there was no statistically significant (Mann–Whitney U test) differences detected between patients with or without another autoimmune disease (AID) (Figure 3A & C), although a few subjects with high concentrations had another AID. No statistically significant (Mann–Whitney U test) differences were detected between patients at high and low risk of developing lymphoma (Figure 3B & D).

Sensitivity and reproducibility of S100A8/ A9 immunoassay

The accuracy of the ELISA was tested for whole mouth saliva (n=1) and parotid saliva (n=2) by spiking samples with a known concentration of S100A8/A9. The mean recovery (accuracy) of the three samples was 107.7% and ranged between 80.5 and 142.9%. The linearity was reported by the manufacturer (spiked whole mouth saliva) (n=4) with a range of 92-115% for dilutions 1:2, 1:4, 1:8 and 1:16 (Supplementary material, Figure S2).

The reproducibility (intra-assay precision) of ELISA was determined as the mean coefficient of variation (CV) of five replicates of three samples (positive parotid saliva) in one assay (Table 1.) The inter-assay precision was 8.2 % using two samples tested on six separate plates, these were comparable to the values reported by the manufacturer (2.7- 4.5% for the intra- assay CV and 3.2- 5.8% for the inter- assay CV).

Associations of S100A8/A9 levels in whole mouth and parotid saliva and their relation to their flow rates

There was a weak positive correlation between the salivary levels of S100A8/A9 in matched parotid and WMS from the same patients (n=56; Spearman r=0.268, p=0.046; Figure 4.). A stronger correlation was found when only SS sub-groups were analysed (n=26) (Spearman r=0.496, p=0.01). A significant correlation was not found in samples from healthy control subjects (n=18; Spearman r=0.106, p=0.67). There was a negative (inverse) correlation between the parotid salivary levels of S100A8/A9 and parotid flow rates (Spearman r=0.256, p=0.02) but not with whole mouth saliva flow rates (Spearman r=0.138, p=0.309).

Discussion

Results from the pilot proteomics analysis of parotid saliva suggested that levels of S100A8 (Calgranulin A) and S100A9 (Calgranulin B) not only discriminate between SS and healthy controls, but also between patients with SS MALT-L and those without lymphoma. This suggests the possibility that S100A8/A9 might act as a biomarker for the development of lymphoma. S100-A8 and S100-A9 are myeloid-related pro-inflammatory members of the Alarmin family and are predominantly found as a S100A8/A9 24 kD heterodimer that plays a prominent role in the regulation of chronic and acute inflammation, inducing leukocyte chemotaxis and adhesion. S100A8/A9 has been reported as being up-regulated in different cancers [15,16] and increased S100A9 protein expression is linked with autoimmune and inflammatory diseases such as rheumatoid arthritis, systemic sclerosis and systemic lupus erythematosus[17].

Results from the subsequent immunoassay of larger numbers of parotid salivas supported and extended the proteomics findings, showing increased expression of S100A8/A9 in parotid saliva of SS groups compared with both healthy controls and non-SS dry mouth (SNOX) disease controls. A higher proportion of parotid salivas from the MALT-L associated SS groups showed concentrations above 2000 ng/mL but the median values were not statistically significantly different from the SS group without MALT-L association. Interestingly it was noted that when comparing left and right glands of two patients at risk of developing MALT-L, the swollen glands showed increased level of S100A8/A9 compared to the contralateral unswollen gland, which

suggested that increased S100A8/A9 expression reflects a localised inflammatory activity. Levels of S100A8/A9 in WMS were higher than parotid saliva but did not show statistically significant increases in SS compared with the healthy and disease controls. A possible explanation is that despite being strict in selecting age-matched healthy controls who were not on any xerostogenic medication, it might be that other influences on oral health status (e.g. caries) affected S100A8/A9 levels in whole mouth saliva. Other sources, in particular gingival crevicular fluid, contribute to S100A8/A9 in WMS and it should be noted that almost a third of the SS subjects were not able to produce whole mouth saliva. Sub-grouping of SS has revealed significant differences in whole mouth S100A8/A9 levels of MALT-L patients when compared to the other sub-groups. This may be related to the important link of S100A8/A9 to cancer as an amplifier of inflammation associated tumour development, which has been previously reported[15,16]. Since the total protein concentration of whole mouth saliva tended to increase in SS associated with MALT-L compared to other groups a comparison of S100A8/A9 levels between the groups was made after adjusting the values to total protein (ng/mg) but the higher levels in MALT-L remained significant.

The results from previous studies [18-23] of salivary S100A8/A9 levels in healthy control and SS subjects are summarised in Table 2. Most previously published studies in SS have assayed stimulated WMS and the mean values in control subjects are higher than the present study, which utilized unstimulated WMS. Readings comparable to those of the present study were obtained by Sweet *et al.* showing a wide range of S100A8/A9 levels in unstimulated WMS (200-18000 ng/mL) but significantly higher concentrations in SS patients[18], a finding in contrast to the present study. While Cuida *et al.* did not show a significant difference in parotid salivary S100A8/A9 between SS and control subjects they did report a positive correlation between parotid salivary levels and minor salivary gland focus scores of SS patients[23]. In our study, a group of non-SS sicca disease control subjects diagnosed with non-specific sialadenitis, nodal osteoarthritis & xerostomia (SNOX) was included. SNOX has been little studied since its first description by Kassimos *et al*., [14] and later by others [24,25] it was therefore of interest to follow this disease group further. SNOX is often confused with SS and is sometimes incorrectly called pre-SS and shares some symptoms; fatigue, complaints and signs of dry mouth and dry eyes; inflammation of the minor salivary glands (non-specific vs. focal in SS groups). Levels of S100A8/A9 in the SNOX group were not significantly elevated in parotid saliva and WMS compared with healthy control

subjects, which suggested that the sialadenitis and degree of parotid inflammation in SNOX patients is less than in SS patients.

A potential limitation of the present study is that some patients with MALT-L were receiving immunosuppressive therapy. However, a comparison of treated and untreated patients did not show a difference in mean S100A8/A9 levels (Supplementary material, Figure S3). This might also be due to insufficient power of the study to determine an effect of drugs in this heterogeneous group of patients. Furthermore, the small sample size of the proteomics analysis should be noted and it would be of great significance to conduct the same comparison on a larger group of patients to check for the reproducibility of the results.

This study supports previous reports indicating the value of saliva as a diagnostic tool for assessing disease activity and progression in Sjögren's syndrome. The preliminary results of parotid saliva proteomic analyses provided a list of candidate biomarkers of SS and SS at risk of lymphoma. It may be that a combination of S100A8/A9 with another parotid salivary molecular or other biomarker, for example parotid ultrasonography (Jazzar *et al.,* submitted), could provide a more robust diagnostic tool.

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Conflict of interest Statement

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Figure legends:

Figure 1. Whole mouth saliva flow rate (A), its total protein concentration (C) (n=56), parotid saliva flow rate (E) and its total protein concentration (G) (n=83) of different groups. (A & C) and (E & G) SS groups are combined (B & D) and (F & H) SS sub-groups.
CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, at risk; at risk of MALT-L. Data are reported as median ± (IQR) and expressed as mL/min.
(Kruskal-Wallis test followed by Dunn's post hoc test).

Figure 2. The most over expressed proteins in SS patients compared to control subjects, as determined by proteomics analysis of parotid saliva samples. (A) Three proteins in the first run *, (B) Four proteins in the second run. The process of selection involved comparison of relative expression levels (fold change ratio) of proteins identified by proteomics analysis between control samples and samples from patients with SS at risk of developing MALT-L and between control samples and SS samples. A secondary comparison of proteins was done after log (log2) transformation of the raw ratios. A final comparison between both runs was performed following their individual analysis where differences between both disease samples (SS and SS at risk) were identified
SS; Sjögren's syndrome, MALT-L risk; SS patients at risk of developing mucosa associated lymphoid tissue lymphoma. * Protein S100-A9 was not detected in the control sample.

Figure 3. Concentration of S100A8/A9 in parotid saliva (A & B) (n=83) and whole mouth saliva (C & D) (n=56) from different groups. (A & C) SS groups are combined (B & D) SS sub-groups.
CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, at risk; at risk of MALT-L, -/+ with or without, AID; autoimmune disease.
Data are reported as median ± (IQR) and expressed as ng/mL.
(Kruskal-Wallis test followed by Dunn's post hoc test).

Figure 4. Spearman rank correlations of S100A8/A9 levels from parotid and whole saliva of all subjects (n=56). CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjögren's syndrome.

Table 1. Intra-assay precision of S100A8/A9 ELISA assay tested on parotid saliva

	Sample1	Sample2	Sample3	Overall mean
Measurements (n)	5	5	5	-
Mean (ng/mL)	3550.3	7268.9	2034.4	4284.5
Standard deviation	124.6	313.9	59.6	166.03
CV (%)	3.5	4.3	2.9	3.9

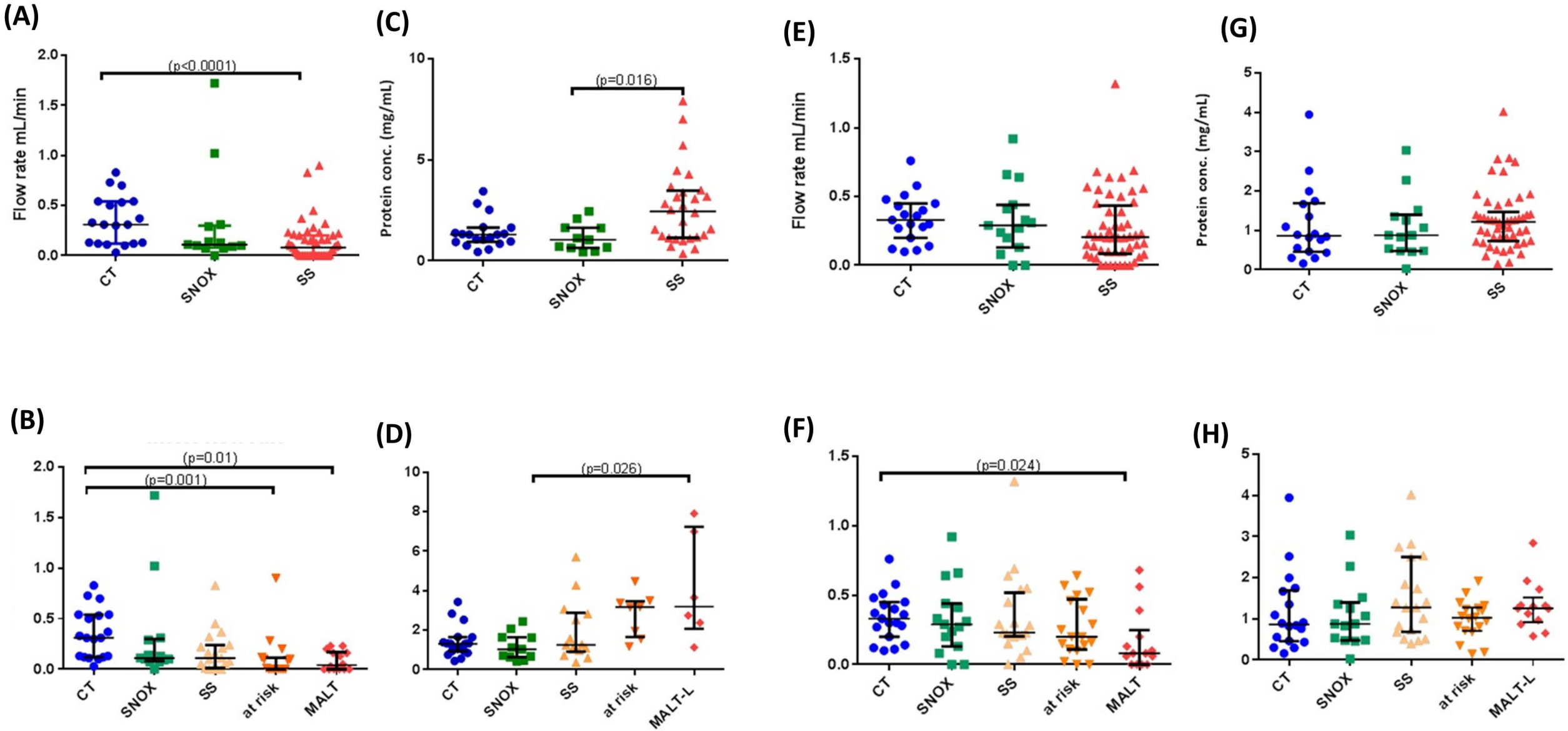
Table 2. Summary of salivary levels of S100A8/A9 in the published literature

Study references	saliva	Mean CT levels(ng/mL)	Mean SS levels(ng/mL)	Significant
Sweet et al. 2001[18]	sParotid	300	-	-
	uWhole	2000	14000	Yes
Muller at al.1992[19]	sParotid	207 *	-	-
Cuida et al.1993[20]	sWhole	27100	29000	No
Brun et al.1994[21]	sWhole	27100	23624	No
Cuida et al. 1995[22]	uParotid	3200	-	-
	sWhole	22000	-	-
Cuida et al. 1997[23]	sParotid	2700	5500	No
	sWhole	15200	36300	No
our results	sParotid	251.4	1968.8	Yes
	uWhole	9545.2	13401	No

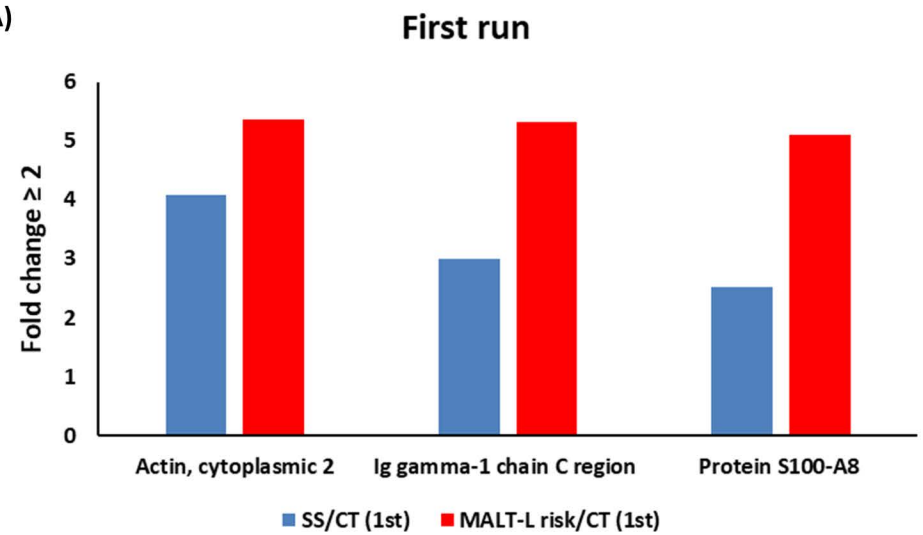
*median, uWhole; unstimulated whole saliva, sWhole; stimulated whole saliva, sParotid; stimulated parotid saliva, uParotid; unstimulated parotid saliva. CT; healthy controls, SS; Sjögren's syndrome.

Whole mouth saliva

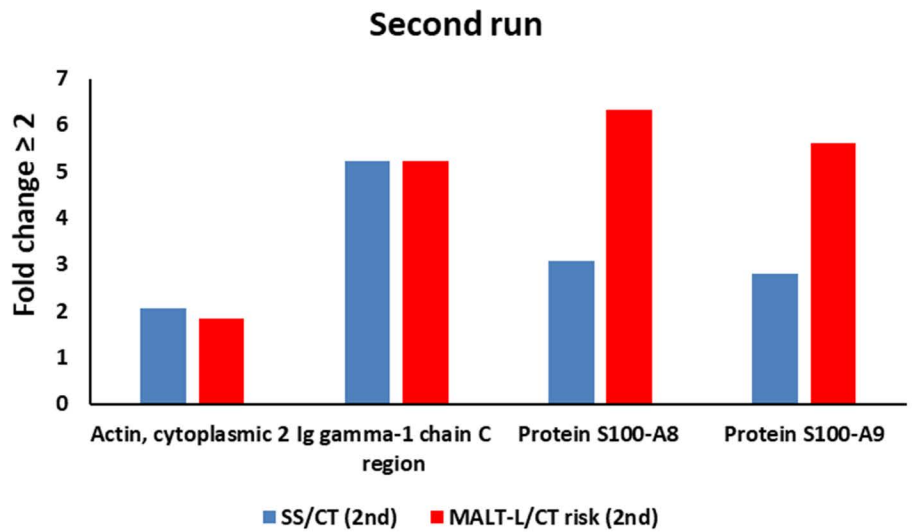
Parotid saliva



A)

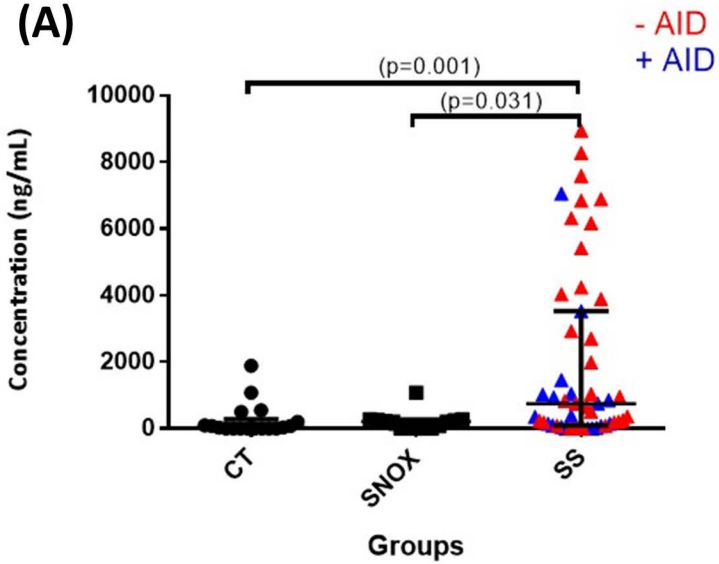

First run
Fold change ≥ 2
0
1
2
3
4
5
6
Actin, cytoplasmic 2
Ig gamma-1 chain C region
Protein S100-A8
SS/CT (1st)
MALT-L risk/CT (1st)

B)

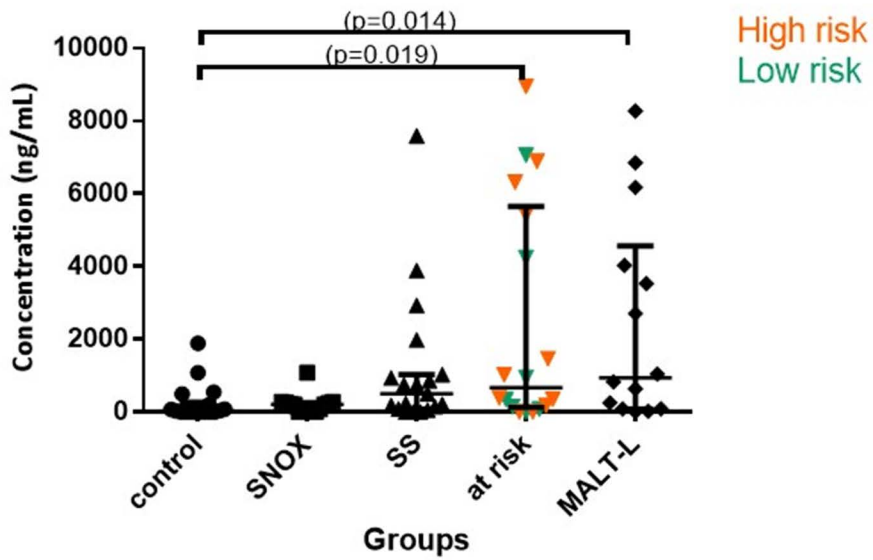

Second run
Fold change ≥ 2
0
1
2
3
4
5
6
7
Actin, cytoplasmic 2
Ig gamma-1 chain C region
Protein S100-A8
Protein S100-A9
SS/CT (2nd)
MALT-L/CT risk (2nd)

Parotid saliva

(A)

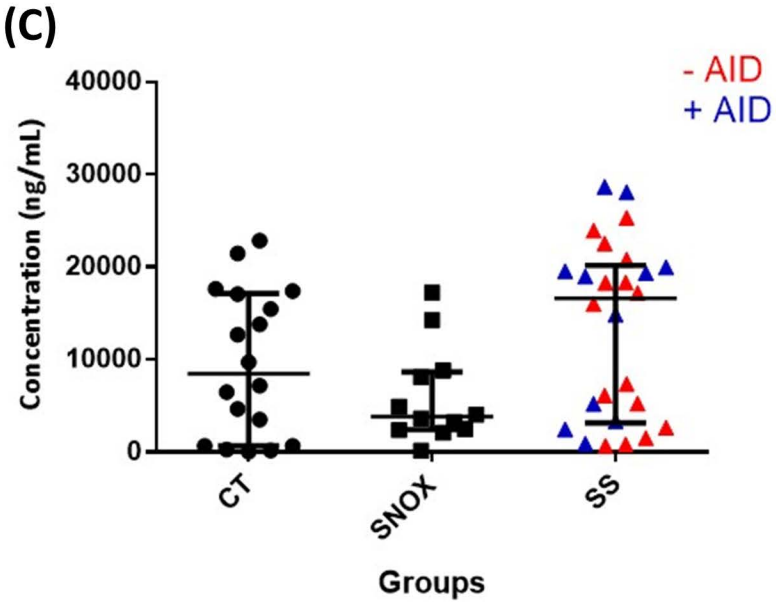


(B)

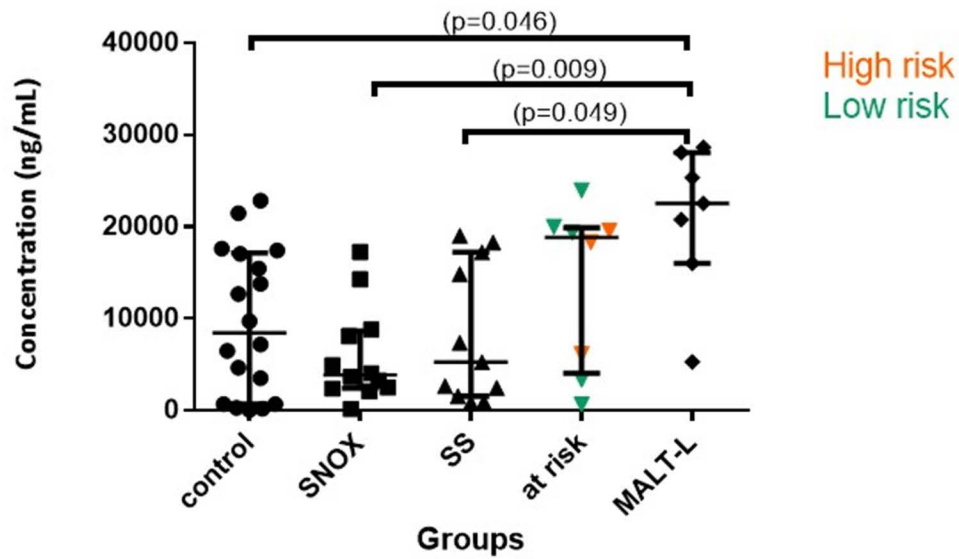


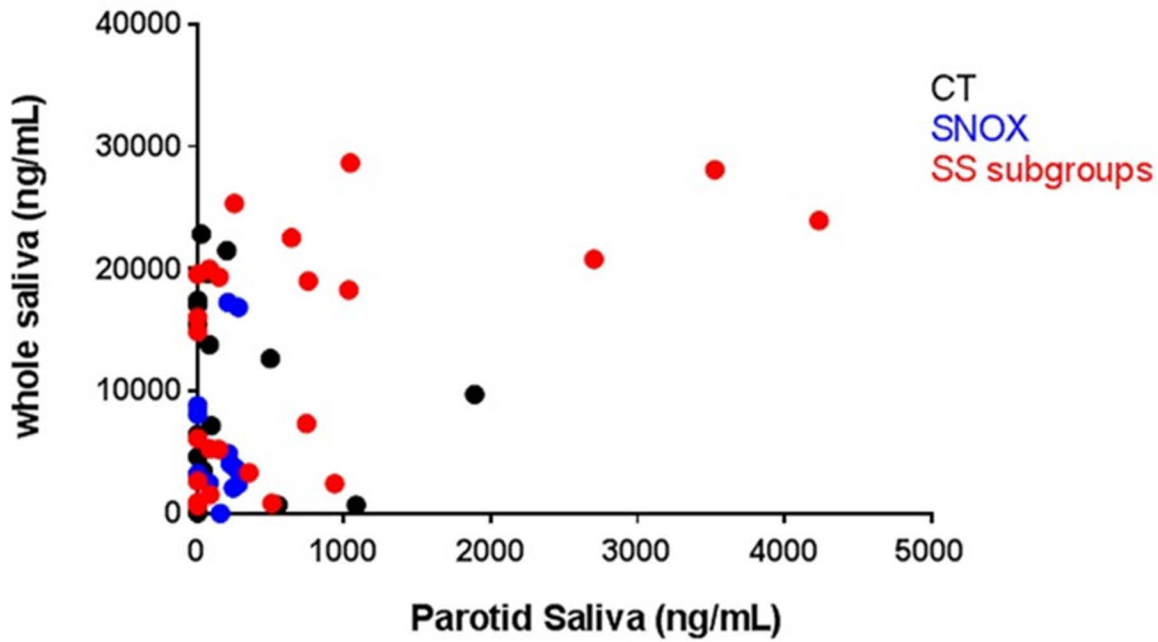
Whole mouth saliva

(C)



(D)




40000
30000
20000
10000
0
whole saliva (ng/mL)
0
1000
2000
3000
4000
5000
Parotid Saliva (ng/mL)
CT
SNOX
SS subgroups